REMARKS

In the Office Action, the Examiner withdrew the anticipation rejection against claims 1-5 and 7-10 over Horrobin et al. (US 5,603,959), maintained the rejection against claims 1-5 and 7-12 under 35 U.S.C. §102(b) as being anticipated by Horrobin et al. (US 6,245,811), maintained the rejection against claims 1-13 under 35 U.S.C. §102(b) as being anticipated by Menard et al. (WO 02/09725), and maintained the rejection against claims 1-16 under 35 U.S.C. §103(a) as being obvious over Cook et al. (US 6,077,868) in view of Watkins et al. (Journal of the American College of Nutrition 2000, 19:478S-486S). Each maintained rejection is addressed separately below. In view of the amendments noted above and the remarks below, applicants respectfully request reconsideration of the merits of this patent application.

No extension of time is believed to be necessary and no fee is believed to be due in connection with this response. However, if any extension of time is required in this or any subsequent response, please consider this to be a petition for the appropriate extension and a request to charge the petition fee to Deposit Account No. 17-0055. No other fee is believed to be due in connection with this response. However, if any fee is due in this or any subsequent response, please charge the fee to the same Deposit Account No. 17-0055.

Anticipation rejection under 35 U.S.C. §102 (b) based on Horrobin et al. (US 6,245,811)

The Examiner rejected claims 1-5 and 7-12 as being anticipated by Horrobin et al. (US 6,245,811). In particular, the Examiner alleged that the document disclosed a method for treating a disorder (rheumatoid arthritis) comprising administering to a patient in need thereof an effective amount of the compound according to claim 1 where R_1 is an acyl moiety corresponding to an acid (conjugated linoleic acid, CLA), citing column 14 at lines 55-62, column 15 at line 1, and claims 1, 10, and 28. Further, the Examiner asserts that said compound is an ester of CLA. Applicants respectfully traverse the rejection in that Horrobin et al. (US 6,245,811) do not specifically disclose that CLA can be used to treat rheumatoid arthritis.

The U.S. patent issued to Horrobin et al. (US 6,245,811) is not about new activities of any compound. Rather, it is about combining two or more known bioactive compounds into the same molecule to achieve increased lipophilicity (for passing lipid barriers in the body) as well as additive or maybe even synergistic effect (col.1, lines 13-64). In this regard, it relies on

compounds with known bioactivities. At the time the Horrobin et al. application was filed (August 18, 1999), CLA was known to be effective for treating some other diseases but not rheumatoid arthritis. The specification of Horrobin et al. is consistent with this. For example, Horrobin et al. mentions the treatment of rheumatoid arthritis in connection with one or more of GLA, DGLA, SA and EPA but not CLA (see column 13, lines 12-16 and 30-31). The only activities specifically mentioned in connection with CLA are, as known in the art at that time, treating or preventing cancer, treating or preventing cardiovascular disease, treating metabolism diseases, promoting growth of protein-containing tissues, and acting as an antioxidant (see column 6, lines 35-39 and column 14, lines 6-10). Horrobin et al. did not present any data to indicate that they discovered a new activity of CLA for treating rheumatoid arthritis.

The sections of Horrobin et al. (US 6,245,811) cited by the Examiner (col. 14 at lines 55-62, col. 15 at line 1, and claims 1, 10, and 28) list a plurality of compounds including among others GLA, DGLA, SA, EPA, DHA, and CLA for treating a plurality of diseases including among others impotence, male pattern baldness, renal and urinary tract disorders, cancer, cardiovascular disease/disorder, and rheumatoid arthritis (col. 14 at lines 55-62 and 66, col. 15 at lines 1 and 9, and claims 1, 10, and 28). The Examiner seems to read the above sections to mean that any of the compounds listed can treat any of the diseases listed. However, this is not the case. For example, EPA and DHA which are among the bioactive compounds listed are not effective for treating impotence and male pattern baldness which are among the diseases listed, although they are effective for treating some other listed diseases such as renal and urinary tract disorders. Similarly, CLA was not known to be effective for treating rheumatoid arthritis but known to be effective for treating other listed diseases such as cancer and cardiovascular disease. Therefore, the reasonable way of reading of the sections cited by the Examiner would be that a compound can be used to treat one or more diseases listed there for which the compound was known to be effective at the time the Horrobin et al. application was filed but not that a compound can be used to treat all of the diseases listed. This reading does not violate any claim construction rules with respect to claims 1, 10, and 28 cited by the Examiner. Accordingly, given that Horrobin et al. did not specifically disclose or present any data to indicate that they had identified a new activity of CLA for treating rheumatoid arthritis, a skilled artisan would understand that CLA was listed among the bioactive compounds for its known effects on cancer

and cardiovascular disease, but not rheumatoid arthritis for which one or more other compounds that were known to be effective were among the compounds listed. .

For the above reasons, applicants respectfully submit that claims 1-5 and 7-12 are not anticipated by Horrobin et al. (US 6,245,811).

Anticipation rejection under 35 U.S.C. §102 (b) based on Menard et al. (WO 02/09725)

The Examiner rejected claims 1-13 as being anticipated by Menard et al. (WO 02/09725). In particular, the Examiner alleged that the document disclosed the treatment of degenerative joint diseases and rheumatoid arthritis by using a composition comprising CLA, glucosamine and ascorbic acid. Without agreeing with the rejection, applicants have amended claim 1 to facilitate prosecution and reserve the right to pursue the canceled subject matter in a continuation application.

Claim 1 as amended recites the phrase "consists of" to limit the therapeutically active component in the composition to only CLA. Since Menard et al. specifically provide throughout the specification and claims that the treatment compositions disclosed therein require three active components (namely, CLA, glucosamine and ascorbic acid), claim 1 and its dependents as amended are not anticipated by Menard et al.

Obviousness rejection under 35 U.S.C. §103 (a)

The Examiner rejected claims 1-16 as being obvious over Cook et al. (US 6,077,868) in view of Watkins et al. (Journal of the American College of Nutrition 2000, 19:478S-486S). In particular, the Examiner alleged that Cook et al. disclose a method of inhibiting cyclooxygenase 2 (COX-2) activity and reducing COX-2-mediated inflammation by using CLA. Further, the Examiner alleged that Watkins et al. disclose that (1) anti-inflammatory diets are associated with decreased pathogenesis of rheumatoid arthritis, reduced inflammatory diseases and lowered cancer risk, (2) up-regulation of COX-2 contributes to inflammation, and (3) the beneficial anti-cancer effect of CLA is likely linked to down-regulation of COX-2 activity. In the Examiner's opinion, it would have been obvious to one of ordinary skill in the art, in view of the two references, to administer CLA for treating rheumatoid arthritis. In this regard, the Examiner asserts that one of ordinary skill in the art would be motivated to do the above, the practice of the

invention of Cook et al. (US 6,077,868) would intrinsically treat rheumatoid arthritis, and there would be reasonable likelihood of success. Applicants respectfully traverse the rejection.

When evaluating the obviousness of a particular invention, the law requires considering the "whole" of the prior art. *See In re Keller*, 642 F.2d 413, 425 (CCPA 1981) (determining obviousness from "what the combined teachings of the references would have suggested to those of ordinary skill in the art"). "When prior art contains apparently conflicting references, the [PTO] must weigh each reference for its power to suggest solutions to an artisan of ordinary skill." *See In re Young*, 927 F.2d 588, 591 (Fed. Cir. 1991).

The two references cited by the Examiner, Cook et al. and Watkins et al., only present part of the prior art story on rheumatoid arthritis. As the applicants have argued in the previous response based partly on another part of the prior art (illustrated by the Yang et al. reference cited by the applicants), it is counterintuitive to try to use a compound like CLA, which has been indicated to be able to increase antibody production, to treat rheumatoid arthritis. Applicants discuss this in detail below with additional supporting references.

Cook et al. and Watkins et al. focus on the CLA's inhibitory effect on COX-2 and the synthesis of prostaglandins by COX-2 that causes inflammation and pain. However, this is only part of the story for rheumatoid arthritis. As discussed in the present application (e.g., paragraphs [00010] and [00011]), type III hypersensitivity is caused by antibody/antigen immune complex deposition, leading to tissue damage and inflammatory reactions. In the case of rheumatoid arthritis, the immune complex is between the auto-antibodies to type II collagen and type II collagen. Therefore, prostaglandins production and inflammation reactions are only secondary targets for treating type III hypersensitivities such as rheumatoid arthritis. Given that antibody-initiated type III hypersensitivities depend on the production of antibody (the primary cause of type III hypersensitivities), it would be counterintuitive to try to use an agent that can increase antibody production for the treatment of type III hypersensitivities such as rheumatoid arthritis.

At the time the application was filed, there was evidence in the art that CLA can increase antibody production in the body. For example, Sugano et al. have shown that CLA increases immunoglobulin (antibody) production (M. Sugano et al., Lipids, 1998, 33:521-527, copy attached). Similarly, Yamasaki et al. have also shown that CLA increases immunoglobulin (antibody) production (M. Yamasaki et al., J. Nutr, 2003, 133:784-788, copy attached).

Furthermore, Yang et al. studied the effect of CLA on another auto-antibody immune complex disease, systemic lupus erythematosus, and showed that CLA treatment promoted the earlier appearance of antinuclear antibodies as well as proteinuria (the first clinical sign of renal failure in Lupus due to antinuclear antibody immune complexes). See Figs. 3 and 4 of Yang et al., Immunopharmacology and Immunotoxicology, 2000, 22:433-449 (copy submitted in connection with the previous response). This body of evidence indicates that CLA may aggravate rather than reduce antibody-initiated type III hypersensitivities that are caused by immune complex deposit.

For a disease such as osteoarthritis wherein a trauma (rather than antibody/antigen immune complex) causes tissue damage that leads to an inflammatory response involving prostaglandins, an argument may be made that a skilled artisan might try to use CLA to treat the disease based on the CLA's inhibitory effect on COX-2 activity and the fact that CLA would not aggravate the primary source of tissue damage. However, it would be counterintuitive to try to use CLA to treat type III hypersensitivities such as rheumatoid arthritis wherein CLA may aggravate the disease by increasing the production of a component (antibody) of primary cause of the disease.

As discussed above, the law requires considering the "whole" of the prior art in the context of obviousness inquiry. When the three references on CLA's effects on antibody production cited above by the applicants are considered, at the very least, they would introduce sufficient doubt into the mind of a skilled artisan so that a skilled artisan would not have concluded that there would be a reasonable likelihood of success for using CLA to treat type III hypersensitivities such as rheumatoid arthritis.

In response to the applicants' reply to the Examiner's point that practicing the invention of Cook et al. (US 6,077,868) would *intrinsically* treat rheumatoid arthritis, the Examiner cited *Atlas Powder Co. v. Ireco Inc.* (190 F.3d 1342, 1347 (Fed. Cir. 1999)) for the proposition that "the discovery of a previous unappreciated property of a prior art composition, or of a scientific explanation for the prior art's functioning, does not render the old composition patentably new to the discoverer." However, the claims at issue are not directed at old compositions based on some new properties that the inventors discovered. Rather, they are directed at a method of using an old agent based on the newly discovered activity of the agent. Applicants acknowledge the *In re Best* case (562 F.2d 1252, 1254 (CCPA 1977)) cited by the Examiner. As the Examiner pointed

out, the court in *In re Best* said that the claiming of a new use, new function or unknown property, which is inherently present in the prior art does not necessarily make the claim patentable. Applicants respectfully note that by using the word "necessarily," the court indicates that the claiming of a new use may sometimes be patentable.

Perricone v. Medicis Pharmaceutical Corp., 432 F.3d 1368 (Fed. Cir. 2005) presents a parallel fact pattern to the present application. In *Perricone v. Medicis Pharmaceutical Corp.*, the Federal Circuit was presented with the question whether a claim (claim 1 in U.S. patent 5,574,063) is anticipated under the doctrine of inherency wherein the claim is directed at a method for treating skin sunburn comprising topically applying to the skin sunburn a composition the active ingredients of which have been disclosed by a prior art reference (U.S. patent 4,981,846) in connection with skin cream compositions. The argument made for invalidating the claim is that skin sunburn would be inherently treated by the prior art skin creams. However, the Federal Circuit held that the claim is not anticipated by the prior art reference because the correct inquiry is not whether the prior art cream if applied to skin sunburn would inherently treat the damage but whether the prior art reference discloses applying the skin cream to sunburn and it does not. Similarly, for the resent application, the correct question to ask is not whether CLA if administered to someone who has a type III hypersensitivity-related disease or condition would inherently treat the disease or condition but whether Cook et al. disclosed administering CLA to a human or non-human animal who has a type III hypersensitivity-related disease or condition. In this regard, Cook et al. did not make the disclosure. Therefore, the claims at issue are not inherently anticipated by Cook et al. In this regard, claim 1 has been amended to clarify that CLA is administered to a human or non-human animal in need of having a type III hypersensitivity-caused disease or condition treated.

As an aside, without agreeing that practicing the invention of Cook et al. (US 6,077,868) would *intrinsically* treat rheumatoid arthritis, applicants respectfully note that what is inherent in the prior art, if not known at the time of the invention, cannot form a proper basis for rejecting the claimed invention as obvious under § 103. *See In re Shetty*, 566 F.2d 81, 86 (CCPA 1977).

For the above reasons, applicants respectfully submit that, at the time the present application was filed, the evidence from the art when considered as a whole would not motivate a skilled artisan to try to use CLA to treat rheumatoid arthritis because CLA had been shown to increase the production of antibody, a component of the primary cause of rheumatoid arthritis.

Even for the sake of argument that one would try said treatment, there would not have been any reasonable likelihood of success for the same reasons.

Conclusion

Having addressed each rejection maintained by the Examiner, the claims as amended are believed to be in condition for allowance and a Notice of Allowance is respectfully requested. Should any issues remain outstanding, the Examiner is invited to contact the undersigned at the telephone number appearing below if such would advance the prosecution of this application.

Respectfully submitted,

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Immunoglobulin and Cytokine Production from Spleen Lymphocytes Is Modulated in C57BL/6J Mice by Dietary *Cis*-9, *Trans*-11 and *Trans*-10, *Cis*-12 Conjugated Linoleic Acid

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ABSTRACT We evaluated the effect of *cis*-9, *trans*-11 (9*c*,11*t*) and *trans*-10, *cis*-12 (10*t*,12*c*) conjugated linoleic acid (CLA) on the immune system in C57BL/6J mice. Mice were fed experimental diets containing 0% CLA (controls), 1% 9*c*,11*t*-CLA, 1% 10*t*,12*c*-CLA or a 1:1 mixture (0.5% + 0.5%) of these two CLA isomers for 3 wk. Relative spleen weights of all CLA fed mice were greater than the controls. Spleen lymphocytes isolated from the mice fed 10*t*,12*c*-CLA produced more immunoglobulin (Ig)A and IgM but not IgG when stimulated with concanavalin A (ConA) compared with controls. IgA production from unstimulated spleen lymphocytes was greater in the 10*t*,12*c*-CLA group than in controls. Conversely, 9*c*,11*t*-CLA did not affect the production of any of the Ig subclasses. Lymphocytes isolated from 9*c*,11*t*-CLA fed mice produced more tumor necrosis factor-α than the control group. The proportion of B cells in the spleen lymphocyte population was significantly lower in the 9*c*,11*t*-CLA group, and higher in the 10*t*,12*c*-CLA group than in the controls. Compared with the control group, the percentage of $CD4^+$ T cells was lower in the 10*t*,12*c*-CLA group, and the percentage of $CD8^+$ T cells was higher in the 9*c*,11*t*-CLA group. Furthermore, the percentage of $CD8^+$ T cells was higher in the 1:1 mixture group than in controls. The $CD4^+/CD8^+$ ratio was lower in the 1:1 mixture group than in controls. These results suggest that 9*c*,11*t* and 10*t*,12*c*-CLA can stimulate different immunological effects and that the simultaneous intake of the two isomers can change the T cell population. J. Nutr. 133: 784–788, 2003.

KEY WORDS: • *conjugated linoleic acid* • *immunoglobulin* • *mice* • *cytokine*

Conjugated linoleic acid (CLA)[2] is a generic term for the positional and structural isomers of octadecadienoic acid. CLA has been reported to exert various beneficial physiologic effects. In previous reports, the effects of CLA were often evaluated using a mixture of CLA isomers, which contained mainly the *cis*-9, *trans*-11 (9*c*,11*t*) and the *trans*-10, *cis*-12 (10*t*,12*c*)-CLA isomers. Recently, the role of each of these CLA isomers has been studied separately. In fact, some differences between the two isomers have been reported, especially the antiobesity effect (1–4). We reported previously that dietary CLA enhanced immunoglobulin (Ig) production in rat spleen and mesenteric lymph node lymphocytes (5,6). Stimulation of Ig production by dietary CLA was detected at an extremely low dietary level (0.5 g/kg diet) (7). However, determining which of the CLA isomers stimulates Ig production and the mechanism of that stimulation remain to be more clearly elucidated. Thus, the objective of this study was to evaluate the individual and simultaneous effect of 9*c*,11*t* and 10*t*,12*c*-CLA on the production of Ig and cytokines and on the population of B and T cells in spleen lymphocytes from C57BL/6J mice.

MATERIALS AND METHODS

Experimental animals and diet. Semipurified 9*c*,11*t* and 10*t*,12*c*-CLA and safflower oil were purchased from Rinoru Oil Mills (Nagoya, Japan). Male 4-wk-old C57BL/6J mice (n = 40) (Japan CLEA, Tokyo, Japan) consumed a nonpurified commercial diet and water ad libitum for 2 wk after their arrival. After acclimation, the mice were divided into 4 groups of 10 mice each. They were kept in the Biotron Institute of Kyushu University with a 12-h light:dark cycle (light, 0800–2000 h) in an air conditioned room (20°C and 60% humidity under specific pathogen–free conditions). This experiment was carried out according to the guidelines for animal experiments at the Faculty of Agriculture and the Graduate Course, Kyushu University, and the Law (No. 105) and Notification (No. 6) of the Japanese Government. The experimental diets were manufactured according to the AIN-93G standard (8) and contained 0 g/kg CLA (control), 5 g/kg 9*c*,11*t* and 5 g/kg 10*t*,12*c*-CLA (1:1 mixture), 10 g/kg 9*c*,11*t* (9*c*,11*t*) or 10*t*,12*c*-CLA (10*t*,12*c*) (**Table 1**). For the basic dietary fat source, we used high linoleic acid safflower oil as in

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[2] Abbreviations used: ConA, concanavalin A; CLA, conjugated linoleic acid; Ig, immunoglobulin; IFN-γ, interferon-γ; IL, interleukin; LPS, lipopolysaccharide; PE, phycoerythrin; POD, peroxidase; TNF-α, tumor necrosis factor-α.

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TABLE 1

Composition of the experimental diet[1]

Ingredient	*g/kg diet*
Corn Starch	397.5
Casein	200.0
Dextrinized corn starch	132.0
Sucrose	100.0
Fat[2]	70.0
Cellulose	50.0
AIN-93G mineral mix	35.0
AIN-93G vitamin mix	10.0
L-Cystine	3.0
Choline bitartrate	2.5
Tert-buthylhydroquinone	0.014

[1] Experimental diet was prepared according to the AIN-93G guidelines (8).
[2] Detailed fatty acid composition in each dietary group is shown in TABLE 2.

our previous report (7). The fatty acid composition of these diets is shown in Table 2. At the end of the feeding period, mice were killed by drawing blood from the abdominal aorta under light anesthesia with diethylether. Immediately after excision, each tissue was weighed and the lymphocytes were isolated from the spleen.

Preparation and cultivation of spleen lymphocytes. Preparation of spleen lymphocytes was performed according to the method described previously (5) without removing adhesive cells such as macrophages and mononuclear cells. First, a cell suspension prepared from the spleen was rinsed with RPMI 1640 medium three times (Nissui, Tokyo, Japan). Then, 5 mL of the cell suspension was added to Lympholyte-mouse (Cedarlane, Hornby, Canada) to isolate the lymphocytes and the suspension was again washed three times with RPMI 1640 medium. The lymphocytes, 2.5×10^9 cells/L, were cultured in RPMI 1640 medium containing 10% fetal bovine serum (Intergen, Purchase, NY) with or without 5 mg/L concanavalin A (ConA) (Sigma, St. Louis, MO) and incubated at 37°C for 24 h.

Measurement of immunoglobulin levels. Measurement of Ig concentration in the cultured medium was performed by a sandwich ELISA. Rabbit anti-mouse IgA (Zymed, San Francisco, CA), goat anti-mouse IgG (H+L) (Zymed), rabbit anti-mouse IgM (μ-chain specific) (Zymed), and anti-mouse IgE from clone LO-ME-3 (heavy-chain specific) (Technopharm Biotechnology, Paris, France) were used to fix each Ig. These antibodies were diluted using 10% Block Ace (Dainihon Pharmaceutical, Osaka, Japan), added to a 96-well plate and incubated for 1 h at 37°C. Then, 300 μL of 10% (25% in IgE) Block Ace was added and kept at 4°C overnight; samples (50 μL) were added to each well for 1 h at 37°C. Each well was treated with a solution of either peroxidase (POD)-conjugated goat anti-mouse IgA (Zymed), POD-conjugated goat anti-mouse IgG (H+L) (Zymed), POD-conjugated rabbit anti-mouse IgM (Zymed), or POD-conjugated goat anti-mouse IgE [GAM/IgE (Fc) PO, Nordic Immunological Laboratory, Tilburg, Netherlands] to detect the respective Ig and incubated for 1 h at 37°C (20 min, 4°C for IgE). The plates were rinsed with PBS containing 0.5 g/L polyethylene sorbitan monolaurate (Nacalai Tesque, Kyoto, Japan) between each step. Then, a 10:9:1 mixture of 1.8 mmol/L H_2O_2 in 0.2 mol/L citrate buffer (pH 4.0), H_2O, and 11.7 mmol/L of 2,2'-azinobis (3-ethylbenzothiazoline sulfonic acid) was added. Finally, absorbance at 415 nm was measured after the addition of 160 mmol/L oxalic acid to stop the coloring reaction.

Measurement of cytokine levels. We measured the level of interleukin (IL)-2, 4, 5, tumor necrosis factor (TNF)-α and interferon (IFN)-γ in the supernatants of spleen lymphocytes cultured with ConA. IL-2, 4 and 5 were measured using commercial ELISA kits following the manufacturers' experimental protocols (Mouse IL-2, 4 and 5 ELISA kit, BioSource International, Camarillo, CA). TNF-α and IFN-γ levels were measured by sandwich ELISA as previously reported (9). Briefly, rabbit anti-mouse/rat IFN-γ (BioSource) and anti-mouse TNF-α (Endogen, Woburn, MA) (500 times dilution) were used to fix IFN-γ and TNF-α for 1 h at 37°C. Then, blocking was performed using 25% Block Ace at 37°C for 1 h. In the following step, 50 μL of appropriate cultured supernatant was added to each well and incubated for 2 h at 37°C; the plate was then treated with either a diluted solution of biotinylated anti-mouse IFN-γ (Genzyme, Cambridge, MA) (500 times dilution) or biotinylated anti-mouse/rat TNF-α (Genzyme) (250 times dilution) for 1 h at 37°C. After that, streptavidin POD-conjugated (Zymed) diluted by 10% Block Ace was added to each well. Plate washing between each step and the coloring reaction were performed as in the Ig measurement protocol.

B and T cell population analysis. The cell surface expression of CD45R (as a B cell marker) and CD4 and CD8 (as T cell subpopulation markers) was analyzed by flow cytometry. After the isolation of lymphocytes from the spleen, cells were washed with RPMI-1640 medium three times and treated with PBS containing 3% bovine serum albumin for 1 h at 37°C. Cells were divided into two groups (1.0×10^6 cells each); one was exposed to rat phycoerythrin (PE)-conjugated monoclonal anti-mouse CD45R (clone RA3-6B2, Caltag Laboratories, Burlingame, CA) and the other was double stained with rat fluorescein isothiocyanate–conjugated monoclonal anti-mouse CD4 (clone CT-CD4, Caltag Laboratories) and rat PE-conjugated monoclonal anti-mouse CD8b (clone CT-CD8b, Caltag Laboratories). All of the antibody reactions were performed on ice for 1 h, and cells were washed three times with PBS after the antibody treatment. Samples were subjected to flow cytometry (FACS Calibur, Becton Dickinson, Sunnyvale, CA) and a total of 10^4 cells were analyzed to determine the percentage of CD45R-, CD4- and CD8-positive lymphocytes.

Statistical Analysis. At first, data were analyzed using one-way (Tables 3, 5 and 6) or two-way (Table 4) ANOVA. The latter was used to identify differences due to diet or lymphocyte stimulation status. Fisher's Protected Least Significant Difference test was used to determine which means differed ($P < 0.05$). All data are presented as means ± SEM.

RESULTS

Food intakes and body and organ weights. Food intake did not differ among the dietary groups (Table 3). Final body weight was higher in the 9*c*,11*t* group than in the 1:1 mixture or 10*t*, 12*c* groups, but it did not differ significantly from the controls. Relative liver weight was greater in the 1:1 mixture and 10*t*,12*c* groups than in the control and 9*c*,11*t* groups. Relative spleen weight was significantly higher in all CLA

TABLE 2

Fatty acid composition of the diets[1]

Groups	Control	1:1 MIX	9*c*, 11*t*	10*t*, 12*c*
	g/100 g total fatty acids			
Fatty acids				
16:0	6.4	5.5	5.4	5.5
18:0	2.5	2.1	2.1	2.1
18:1	15.9	14.6	15.2	13.9
18:2 (n-6)	72.8	62.5	61.6	62.3
CLA				
9*c*, 11*t*	ND	6.2	12.5	0.5
10*t*, 12*c*	ND	6.2	0.8	12.5
9*c*, 11*c*	ND	0.1	ND	0.2
10*c*, 12*c*	ND	0.2	ND	0.3
All *trans*	ND	0.3	0.1	0.5
Others	2.4	2.1	2.1	2.1

[1] High linoleic acid safflower oil was the basic dietary fat source. Experimental diets containing 0 g/kg CLA (control), 5 g/kg 9*c*, 11*t* and 5 g/kg 10*t*, 12*c*-CLA (1:1 MIX), 10 g/kg 9*c*, 11*t* (9*c*, 11*t*) or 10*t*, 12*c*-CLA (10*t*, 12*c*). ND, not detected.

TABLE 3

Food intake, body weight and relative organ weights in C57BL/6J mice fed 0 g/kg CLA, 5 g/kg 9c, 11t and 5 g/kg 10t, 12c-CLA, 10 g/kg 9c, 11t, or 10t, 12c-CLA diet for 3 wk[1]

	Control	1:1 MIX[2]	9c, 11t	10t, 12c	ANOVA
			g/day		
Food intake	3.0 ± 0.0	2.9 ± 0.2	2.9 ± 0.1	2.9 ± 0.1	NS
			g		
Body weight					
Initial	22.7 ± 0.5	22.7 ± 0.4	22.9 ± 0.2	22.6 ± 0.3	NS
Final	26.5 ± 0.5^{ab}	26.0 ± 0.5^{b}	27.7 ± 0.3^{a}	25.6 ± 0.6^{b}	$P < 0.05$
			g/100 g body		
Organ weights					
Liver	42.4 ± 1.0^{b}	51.9 ± 1.0^{a}	44.2 ± 1.1^{b}	56.9 ± 3.2^{a}	$P < 0.001$
Spleen	2.9 ± 0.2^{b}	3.4 ± 0.3^{a}	3.2 ± 0.2^{a}	3.5 ± 0.5^{a}	$P < 0.01$
Lung	6.0 ± 0.1	6.3 ± 0.1	5.7 ± 0.2	6.4 ± 0.2	NS
Heart	5.3 ± 0.1	5.5 ± 0.1	5.6 ± 0.2	5.6 ± 0.2	NS
Kidney	13.3 ± 0.2	13.7 ± 0.1	13.5 ± 0.4	13.2 ± 0.4	NS
PWAT	4.1 ± 0.4^{a}	1.4 ± 0.2^{b}	4.2 ± 0.4^{a}	1.1 ± 0.1^{b}	$P < 0.001$
EWAT	16.1 ± 1.2^{a}	3.5 ± 0.1^{b}	17.1 ± 1.1^{a}	1.8 ± 0.2^{b}	$P < 0.001$

[1] Values are mean ± SEM, $n = 10$. Means in a row not sharing a superscript letter differ, $P < 0.05$. PWAT, perirenal white adipose tissue; EWAT, epididymal white adipose tissue; CLA, conjugated linoleic acid.
[2] The "1:1 MIX" denotes a 1:1 mixture of 9c, 11t and 10t, 12c-CLA.

groups than in the control group. Perirenal and epididymal white adipose tissue weights were significantly lower in the 1:1 mixture and 10t,12c compared with the control and 9c,11t groups. Relative lung, heart and kidney weights did not differ among the dietary groups.

Immunoglobulin production from the spleen lymphocytes. ConA did not affect IgA productivity in spleen lymphocytes (Table 4). IgA productivity of 10t,12c-fed mice was approximately twice that ($P < 0.05$) of the control group in the presence of ConA. IgA productivity also differed between the control and 10t,12c groups without ConA stimulation. IgA production from the lymphocytes in the 9c,11t group was lower than that of the 10t,12c group, irrespective of ConA stimulation. ConA stimulation significantly elevated IgG productivity in spleen lymphocytes. IgM production in spleen lymphocytes was significantly modulated by the type of dietary fat but not by ConA stimulation. IgM production from the lymphocytes in the 10t,12c group was significantly higher than that of the control group, irrespective of ConA stimulation. The 9c,11t and control groups did not differ significantly in IgM productivity. IgM productivity for the 1:1 mixture group was intermediate between the 9c,11t and 10t,12c groups. IgE production by lymphocytes was not affected by diet or ConA stimulation.

Cytokine productivity. Cytokines were not detected in any of the cultured supernatants from lymphocytes that were not stimulated with ConA. No significant differences were found in IL-2, 4, 5 and IFN-γ production among any of the dietary groups (data not shown). TNF-α production of spleen lymphocytes from mice in the 9c,11t group was significantly higher than that of the 10t,12c and control groups (Table 5).

B- and T-cell population. The percentage of B cells in the 9c,11t group was significantly lower than in the control group, whereas the 10t,12c group was higher (Table 6). The value for the 1:1 mixture group was intermediate between the 9c,11t and 10t,12c groups. The percentage of $CD4^+$ T cells was significantly lower in the 1:1 mixture and 10t,12c groups than in the control group. The percentage of $CD8^+$ T cells was the highest for the 1:1 mixture group followed by the 9c,11t group. The $CD4^+/CD8^+$ ratios in the 9c,11t and 10t,12c groups were

TABLE 4

Immunoglobulin A, G, M and E production of spleen lymphocytes isolated from C57BL/6J mice fed 0 g/kg CLA, 5 g/kg 9c, 11t and 5 g/kg 10t, 12c-CLA, 10 g/kg 9c, 11t, or 10t, 12c-CLA diet for 3 wk[1]

	Control	1:1 MIX[2]	9c, 11t	10t, 12c
		ConA (+),[3] μg/L		
IgA	3.0 ± 0.4^{a}	3.4 ± 0.5^{ab}	2.8 ± 1.6^{a}	6.2 ± 1.7^{b}
IgG	35.0 ± 13.5	31.1 ± 4.0	31.1 ± 6.1	32.9 ± 7.6
IgM	10.7 ± 2.6^{a}	13.4 ± 1.4^{ab}	10.9 ± 2.5^{a}	19.0 ± 3.1^{b}
IgE	18.4 ± 0.7	17.0 ± 1.2	19.4 ± 0.4	18.3 ± 0.7
		ConA (−), μg/L		
IgA	2.1 ± 0.4^{ab}	2.0 ± 0.2^{ab}	1.3 ± 0.8^{a}	4.6 ± 1.5^{b}
IgG	19.2 ± 5.2*	15.7 ± 4.6*	20.2 ± 5.3	31.2 ± 8.5
IgM	12.1 ± 2.8^{a}	15.3 ± 2.4^{ab}	13.7 ± 3.9^{ab}	22.7 ± 4.2^{b}
IgE	17.4 ± 1.0	18.3 ± 1.3	18.4 ± 0.5	18.9 ± 0.5

	Two-way ANOVA			
	IgA	IgG	IgM	IgE
ConA	NS	$P < 0.05$	NS	NS
Diet	$P < 0.05$	NS	$P < 0.05$	NS
Interaction	NS	NS	NS	NS

[1] Values are mean ± SEM, $n = 5$. Means in a row not sharing superscript letter differ, $P < 0.05$. * Different from Con A-stimulated, $P < 0.05$.
[2] The "1:1 MIX" means a 1:1 mixture of 9c, 11t and 10t, 12c-conjugated linoleic acid.

TABLE 5

Tumor necrosis factor-α production of spleen lymphocytes isolated from C57BL/6J mice fed 0 g/kg CLA, 5 g/kg 9c, 11t and 5 g/kg 10t, 12c-CLA, 10 g/kg 9c, 11t, or 10t, 12c-CLA diet for 3 wk[1]

	Control	1:1 MIX[2]	9c, 11t	10t, 12c	ANOVA
			ng/L		
TNF-α	411 ± 68[a]	474 ± 32[ab]	574 ± 76[b]	388 ± 21[a]	$P < 0.05$

[1] Values are mean ± SEM, $n = 5$. Means in a row not sharing a superscript letter differ, $P < 0.05$.
[2] The "1:1 MIX" denotes a 1:1 mixture of 9c, 11t and 10t, 12c-conjugated linoleic acid.

lower than in the control group, and the ratio in the 1:1 mixture group was lower than any of the other dietary groups. The $CD4^+/CD8^+$ ratios in the 9c,11t and 10t,12c groups were also significantly lower than the control group.

DISCUSSION

The aim of this study was to evaluate the isomer-specific effect of CLA on the function of spleen lymphocytes in C57BL/6J mice. At present, 9c,11t and 10t,12c CLA are recognized for various beneficial physiologic functions, with each CLA isomer having both individual and synergistic roles in carrying out those functions such as a body fat–reducing effect or a growth-promoting effect. For example, 10t,12c-CLA definitely plays a leading part in reducing body fat (1,10), and we confirmed that this isomer does indeed have a specific body fat–reducing effect in mice (Table 3). On the other hand, 9c,11t-CLA has been reported to promote mouse growth (10), which our present data also support because we found that the final body weight in the 9c,11t group was significantly higher than that in the control group (Table 3). In addition, dietary CLA significantly increased the spleen weight compared with the control group; however, there was no significant difference among the CLA-fed mice. These data suggest that 9c,11t and 10t,12c-CLA have almost the same ability to increase the spleen mass and that no synergistic effect exists between these isomers. In previous reports, CLA feeding did not increase spleen weight in Sprague-Dawley rats (5,7). Thus, we speculate that species specificity exists between rats and mice spleens in terms of sensitivity to dietary CLA.

We reported previously that dietary CLA could enhance IgA, IgG and IgM production from the rat mesenteric lymph node and spleen lymphocytes (5,6). In rat spleen lymphocytes, only a diet containing 0.5 g/kg CLA dramatically promoted IgA, IgG and IgM production (7). However, as far as we know, no report has clearly shown which isomer(s) promote Ig production. In the present study, significant enhancement of IgA and IgM production was detected in the 10t,12c group but not in the 1:1 mixture and 9c,11t groups. This result indicates that 10t,12c-CLA plays a leading part in promoting Ig production. To examine the effect of CLA on the B cell ratio in spleen lymphocytes, we measured the amount of B lymphocyte–specific surface marker CD45R present to determine the positive cell population percentage. As a result, the B-cell percentage in the 10t,12c group was significantly higher than the control level. On the other hand, a decrease in the B-cell percentage was observed in the 9c,11t group, and the level in the 1:1 mixture group was intermediate to the 9c,11t and 10t,12c groups. Judging from these results, the elevation of the B-cell percentage in spleen lymphocytes by 10t,12c-CLA might be counteracted by 9c,11t-CLA. Taken together, it is likely that elevation of the B-cell ratio by 10t,12c-CLA contributes in part to the promotion of Ig production in spleen lymphocytes. However, we must consider that this augmentation of Ig production (~×2) may be due in part to increased production per B cell because the increased number of $CD45R^+$ cells was not doubled.

Significant enhancement of IgA and IgM production was also detected in the 10t,12c group with or without ConA, which is a T lymphocyte–specific mitogen (Table 4). However, IgA and IgM production by spleen lymphocytes did not increase with ConA stimulation. We reported previously that dietary CLA enhanced Ig production in rat spleen and mesenteric lymph node lymphocytes with LPS stimulation which is a B lymphocyte–specific mitogen (6). In a previous report, dietary CLA stimulated IL-2 productivity in mouse spleen lymphocytes or splenocytes (11,12). In this study, neither dietary 9c,11t nor 10t,12c-CLA could inhibit IL-2 production from spleen lymphocytes. In addition, dietary 9c,11t and 10t,12c-CLA did not affect IL-4 and 5 production from spleen lymphocytes stimulated with ConA, and none of these cytokines were detected without ConA. These data indicate that the enhancement of Ig production in spleen lymphocytes by 10t,12c-CLA was not modulated by the stimulation of T lymphocytes during a 24-h incubation period.

Conversely, 9c,11t-CLA significantly stimulated TNF-α production, and this result is consistent with our previous data (9). Turek et al. (13) reported that dietary CLA reduced TNF-α and IL-6 production in rat macrophages, and CLA has been reported to suppress TNF-α related cachexia (14). Conversely, recent reports showed that CLA did not affect TNF-α production in splenocytes isolated from tumor-bearing rats stimulated with *Escherechia coli* endotoxin (15). Unfortu-

TABLE 6

B and T lymphocytes population of spleen lymphocytes isolated from C57BL/6J mice fed 0 g/kg CLA, 5 g/kg 9c, 11t and 5 g/kg 10t, 12c-CLA, 10 g/kg 9c, 11t, or 10t, 12c-CLA diet for 3 wk[1]

	Control	1:1 MIX[2]	9c, 11t	10t, 12c	ANOVA
CD45R+, %	52.6 ± 0.4[c]	55.6 ± 0.5[b]	50.1 ± 0.5[d]	60.2 ± 0.1[a]	$P<0.001$
CD4+, %	21.3 ± 0.4[a]	19.1 ± 0.1[b]	21.8 ± 0.2[a]	18.3 ± 0.2[c]	$P<0.001$
CD8+, %	10.7 ± 0.1[c]	12.7 ± 0.4[a]	11.8 ± 0.2[b]	11.1 ± 0.1[c]	$P<0.01$
CD4+/CD8+	2.0 ± 0.0[a]	1.5 ± 0.1[d]	1.9 ± 0.0[b]	1.7 ± 0.0[c]	$P<0.001$

[1] Values are mean ± SEM, $n = 5$. Means in a row not sharing superscript letter differ, $P < 0.05$.
[2] The "1:1 MIX" denotes a 1:1 mixture of 9c, 11t and 10t, 12c-conjugated linoleic acid.

nately, there is little information concerning isomer specificity in the regulation of TNF-α production. Our present data indicate that 9*c*,11*t* and 10*t*,12*c*-CLA have quite different effects on the production of TNF-α in spleen lymphocytes, but further studies are warranted to elucidate the target immune cells of each CLA isomer.

Most $CD8^+$ T cells are major histocompatibility complex class I restricted killer T cells and exert cytotoxic activity when they are activated. CLA has been reported to elevate the $CD8^+$ T cell population of porcine peripheral mononuclear cells (16,17). Conversely, CLA elevated $CD4^+$ T cell population in mice and chicks (14,18), which resulted in an elevation of the $CD4^+/CD8^+$ ratio. In this report, only 10*t*,12*c*-CLA decreased the $CD4^+$ T cells ratio and only the 9*c*,11*t*-CLA elevated the $CD8^+$ T cells ratio. Interestingly, the $CD4^+/CD8^+$ ratio was the lowest in the 1:1 mixture group. These results suggest that both CLA isomers cooperatively modulated the T cell subpopulation and also acted individually.

In summary, 10*t*,12*c*-CLA increases IgA and IgM production and 9*c*,11*t*-CLA increases TNF-α production. Moreover, these CLA isomers synergistically reduce the $CD4^+/CD8^+$ T cell population ratio.

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Conjugated Linoleic Acid Modulates Tissue Levels of Chemical Mediators and Immunoglobulins in Rats

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ABSTRACT: The effects of conjugated linoleic acid (CLA) on the levels of chemical mediators in peritoneal exudate cells, spleen and lung, and the concentration of immunoglobulins in mesenteric lymph node and splenic lymphocytes and in serum were examined in rats. After feeding diets containing either 0 (control), 0.5 or 1.0% CLA for 3 wk, there was a trend toward a reduction in the release of leukotriene B_4 (LTB_4) from the exudate cells in response to the dietary CLA levels. However, CLA did not appear to affect the release of histamine. A similar dose-response pattern also was observed in splenic LTB_4, lung LTC_4 and serum prostaglandin E_2 levels, and the differences in these indices between the control and 1.0% CLA groups were all statistically significant. The reduction by CLA of the proportions of n-6 polyunsaturated fatty acids in peritoneal exudate cells and splenic lymphocyte total lipids seems to be responsible at least in part for the reduced eicosanoid levels. Splenic levels of immunoglobulin A (IgA), IgG, and IgM increased while those of IgE decreased significantly in animals fed the 1.0% CLA diet. This was reflected in the serum levels of immunoglobulins. The levels of IgA, IgG, and IgM in mesenteric lymph node lymphocytes increased in a dose-dependent manner, while IgE was reduced in those fed the higher CLA intake. However, no differences were seen in the proportion of T-lymphocyte subsets of mesenteric lymph node. These results support the view that CLA mitigates the food-induced allergic reaction.
Lipids 33, 521–527 (1998).

Conjugated linoleic acid (conjugated derivatives of linoleic acid, CLA) exerts diverse physiological effects most of which are favorable to human health. A range of studies has shown a marked alleviating effect of CLA on mammary carcinogenesis (1–4). The mechanism underlying this effect is not yet well understood (5), but continued intake of CLA is not necessarily required for suppression of carcinogenesis (6,7). When considering the diverse effects of CLA, it is reasonable that eicosanoids are involved in the mechanism. The influence of CLA on the metabolic processes leading from linoleic acid to arachidonic acid and, hence, eicosanoids appears to be related to their desirable effects, since CLA tended to reduce the tissue level of prostaglandin E_2 (PGE_2), a putative candidate for a cancer-promoting effect of dietary n-6 polyunsaturated fatty acids (PUFA) (8). In addition, there is a possibility that CLA itself serves as substrate of enzymatic systems for eicosanoid production, as it is shown to undergo desaturation and elongation similar to linoleic acid (9), although it is unknown whether these metabolites could be converted to eicosanoids.

Since the food allergic reaction can readily be modified by the type of dietary PUFA, either n-6 or n-3 (10,11), it is interesting to know if CLA could modify it. The clinical symptom of food allergy is induced by the production of chemical mediators such as histamine and leukotriene (LT) and PG triggered by allergen-specific immunoglobulin (Ig)E (12,13). Our previous studies showed a reduction by CLA of the serum PGE_2 level (8), which is one of the typical chemical mediators in the allergic reaction (12,13). In this context, Belury and Kempa-Steczko (14) showed that CLA reduces the proportion of linoleic acid dose-dependently in hepatic phospholipid and suggested this may result in modified arachidonate-derived eicosanoid production by extrahepatic tissues. More recently, Wong *et al.* (15) reported that CLA modulates certain aspects of the immune defense such as lymphocyte proliferation in mice, although the effect was not always reproduced possibly because of the dependence on the duration of the feeding period. In the present study, we measured the production of chemical mediators and the level of Ig in rats fed different levels of CLA, either 0.5 or 1.0%.

MATERIALS AND METHODS

Preparation of CLA. CLA was prepared according to the method described by Ip *et al.* (16). In brief, 50 g of linoleic acid, purity >99% (Sigma Chemical Co., St. Louis, MO) was dissolved in 290 g of ethylene glycol containing 15 g of NaOH and heated at 180°C for 2 h under nitrogen. After cooling to room temperature, the content was adjusted to pH 4 and extracted with *n*-hexane. The hexane layer was washed with 5% NaCl, dehydrated with 3-A molecular sieves (Nacalai

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Abbreviations: CLA, conjugated linoleic acid; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; LT, leukotriene; MLN, mesenteric lymph node; PEC, peritoneal exudate cells; PG, prostaglandin; POD, peroxidase; PUFA, polyunsaturated fatty acid.

Tesqu, Kyoto, Japan) and dried in a rotary evaporator under nitrogen. The purity of CLA was measured by gas–liquid chromatography (Shimadzu GC-17A, Kyoto, Japan) using a Supelcowax 10 column (0.32 mm × 60 m, film thickness, 0.25 μm; Supelco Inc., Bellefonte, PA). Column temperature was raised from 150 to 220°C at a rate of 4°C/min. The identification of peaks was carried out by the equivalent chain length method (17) and gas chromatography–mass spectrometry (Jeol Auto MS 50, Tokyo, Japan). The purity of CLA preparation was 80.7% with the following composition in percentage: 9*c*,11*t*/9*t*,11*c*, 29.8; 10*t*,12*c*, 29.6; 9*c*,11*c*, 1.3; 10*c*,12*c*, 1.4; 9*t*,11*t*/10*t*,12*t*, 18.6; linoleic acid, 5.6; and others, 13.7.

Animals and diets. The animal experiment adhered to the Kyushu University guidelines for the care and use of laboratory animals. Male, 4-wk-old Sprague-Dawley rats were obtained from Japan Charles River (Atsugi, Japan) and housed individually in a room with controlled temperature and light (20–23°C and lights on 0800–2000 h). After acclimation for 4 d, rats were divided into three groups of 10 rats which were given free access to the experimental diets. The diets were prepared according to the recommendation of the American Institute of Nutrition (AIN-93G diet) (18). The basal diet contained the following ingredients, in g/100 g diet: cornstarch 39.8; casein, 20.0; dextrinized cornstarch, 13.2; sucrose, 10.0; soybean oil, 7.0; AIN-93 mineral mixture, 3.5; AIN-93 vitamin mixture, 1.0; L-cystine, 0.3; choline bitartrate, 0.25; cellulose, 5.0; *tert*-butylhydroquinone, 0.002; and either linoleic acid, 1.0; linoleic acid (Control) and CLA, 0.5 and 0.5; or CLA, 1.0. Thus, LA or CLA was added at the expense of soybean oil in the AIN-93G diet. The fatty acid composition calculated from the composition of individual oils is given in Table 1. Body weight and food intake were recorded every other day. After 3 wk of feeding, five rats were used for collection of the exudate cells and the remaining five rats for other analyses. Blood was withdrawn from the abdominal aorta under light diethyl ether anesthesia and tissues were immediately excised.

Preparation of peritoneal exudate cells (PEC). The method described by Matsuo *et al.* (19) was adopted for the preparation of PEC. Tyrode buffer, consisting of 137 mM NaCl, 2.7 mM KCl, 0.4 mM $NaH_2PO_4 \cdot 2H_2O$, 1 mM $MgCl_2 \cdot 6H_2O$, 12 mM $NaHCO_3$, 1.8 mM $CaCl_2 \cdot 2H_2O$, 5.6 mM D-glucose and 0.1% bovine serum fraction V (Boehringer Mannheim GmbH, Mannheim, Germany), pH 7.4, was injected into the rat peritoneal cavity (6 mL/100 g body weight), and the abdomen was gently massaged for 2 min. Then, the cavity was opened, and the buffer containing PEC was recovered with a plastic pipet. The fluid was centrifuged at 200 × *g* for 5 min at 4°C. The supernatant was discarded and the cell pellet was resuspended in Tyrode buffer.

Measurement of leukotriene B_4 (LTB_4) and histamine. LTB_4 was measured as described elsewhere (20–22). PEC (2×10^6 cells) were suspended in Tyrode buffer containing 5 mM calcium ionophore A23187 (Sigma Chemical Co.). After incubating for 20 min at 37°C, 50 mL of the acetonitrile/methanol mixture (6:5, vol/vol) and 50 ng of PGB_2 (Sigma Chemical Co.), as the internal standard, were added. The mixture was kept at −20°C for 30 min and then centrifuged at 1,000 × *g* for 10 min. The supernatant was filtered through a 4-GV 0.22 μm filter (Millipore Corp., Tokyo, Japan). LTB_4 was measured by reversed-phase high-performance liquid chromatography (HPLC) (SCL-10A; Shimadzu Co., Kyoto, Japan) equipped with an ODS-A column (150 × 6.0 mm, 5 μL particle size; YMC, Kyoto, Japan). A mixture of acetonitrile/methanol/water (30:25:45, by vol) containing 5 mM CH_3COONH_4 and 1 mM disodium EDTA, pH 5.6, was used as a mobile phase with a flow rate of 1.1 mL/min. LTB_4 and PGB_2 were detected by absorbance at 280 nm (SPD-10A; Shimadzu Co.). Quantitation of LTB_4 was achieved by comparing the peak area of LTB_4 with that of PGB_2. Histamine content in the culture supernatant was measured fluorometrically (19,23). The intracellular content of histamine also was measured after disrupting the cells by sonication.

Preparation of spleen and mesenteric lymph node (MLN) lymphocytes. Spleen and MLN were excised immediately after withdrawing blood from the aorta, and the tissues were immersed in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) (24,25). The lymphocytes were rinsed three times with the RPMI 1640 medium and filtered to remove tissue scum. To remove fibroblasts, cell suspension was incubated for 30 min at 37°C. Then, 5 mL of the resulting cell suspension was layered on 4 mL of Lympholyte-Rat (Cedarlane, Hornby, Canada) and centrifuged at 1,500 × *g* for 30 min. The lymphocyte band at the interface was recovered, and the cells were rinsed again. The lymphocytes were cultured in 10% fetal bovine serum (Intergen, Purchase, NY) in RPMI 1640 medium at a cell density of 2.5×10^6 cells/mL with or without 2.5 μg/mL of lipopolysaccharide (Bacto lipopolysaccharide B, *Escherichia coli* 026:B6; Difco Laboratories, Detroit, MI). After incubation at 37°C for 24 and 72 h, the concentrations of IgA, IgG, IgM, and IgE were measured by an enzyme-linked immunosorbent assay (ELISA) (26).

T-cell population analysis. Spleen and MLN lymphocytes were analyzed as $CD4^+$- and $CD8^+$-cells by using fluorescein-labeled mouse anti-CD4 (W3/25, mouse IgG1) or phycoerythrin-labeled mouse anti-CD8 (MRC OX-8, mouse IgG1) (both from Serotec Ltd., Kidlington, Oxford, United King-

TABLE 1
Fatty Acid Composition of Dietary Fat[a]

Fatty acid	Group		
	Control	0.5% CLA	1.0% CLA
16:0	9.1	9.0	9.0
18:0	3.2	3.42	3.2
18:1	20.4	20.3	20.1
CLA	—	6.4	12.9
18:2	59.7	53.6	47.4
18:3	7.5	7.5	7.4

[a]Fatty acid composition was calculated from the composition of individual component fats, soybean oil, linoleic acid, and conjugated linoleic acid (CLA).

dom) (23,25). The stained lymphocytes were fixed with 2% paraformaldehyde and analyzed with the EPICS Profile II flowcytometer (Coulter Electronics Ltd., Luto, United Kingdom).

Measurement of serum and culture supernatant Ig by ELISA. Measurements of total Ig were executed using sandwich ELISA methods (24,25). Goat anti-rat IgE, rabbit anti-rat IgG (Fab')2, goat anti-rat IgM (all from Biosoft, Paris, France), and mouse anti-rat IgA (Zymed Lab, San Francisco, CA) were used to fix respective Ig. These antibodies were diluted 1,000 times with 50 mM carbonate-bicarbonate buffer (pH 9.6), and each well of 96-well plates was treated with 100 μL of the solution for 1 h (2 h for IgA) at 37°C. After blocking with 300 μL of the blocking solution overnight at 4°C, each well was treated with 100 μL of the diluted serum or culture supernatant for 1 h (2 h for IgA) at 37°C. Bound IgA was detected by reacting stepwise with 100 μL of peroxidase (POD)-conjugated rabbit anti-rat IgA (1,000 times dilution; Zymed) at 37°C for 2 h, IgG with 100 μL of POD-conjugated rabbit anti-rat IgG (Fab')2 (2,000 times dilution; Cappel, West Chester, PA), and IgM with 100 μL of POD-conjugated goat anti-rat IgM (1,000 times dilution, Cappel) at 37°C for 1 h. Wells were rinsed three times with Tween 20 in phosphate-buffered saline between each step. After incubation at 37°C for 15 min with 100 μL of 1.5% oxalic acid, absorbance at 415 nm was measured with an MPR-A4i ELISA reader (Tosoh, Tokyo, Japan). The bound IgE was detected by reacting with biotin-conjugated mouse anti-rat IgE (2,000 times dilution; Betyl, Montgomery, TX) followed by POD-conjugated avidin (5,000 times dilution, Zymed Lab) at 37°C for 1 h.

Statistical analysis. Data were analyzed by one-way analysis of variance followed by Duncan's new multiple-range test to identify significant differences (27). Values in the text are means ± SE.

RESULTS

Growth performance and tissue weight. As shown in Table 2, there was no difference in food intake and growth of rats for 3 wk among the groups. Thus, the feed efficiency also was comparable among the groups (mean values 0.41 to 0.42). Among tissues weighed, there was a tendency of increasing liver weight and decreasing perirenal adipose tissue weight by dietary CLA and the difference between the linoleic acid and 1.0% CLA groups was significant.

Release of chemical mediators from PEC. PEC isolated from rats fed linoleic acid or CLA were incubated with or without calcium ionophore A23187, and the concentrations of histamine and LTB_4 were measured in the medium. The content of histamine in the cells also was measured to estimate the cellular histamine contents. As shown in Figure 1, the effect of CLA on the release of histamine in PEC was diverse, and there was no significant difference in any of the parameters measured. However, the amounts of histamine stored in the cells tended to decrease with an increasing dietary level of CLA. There was a trend toward a reduction in

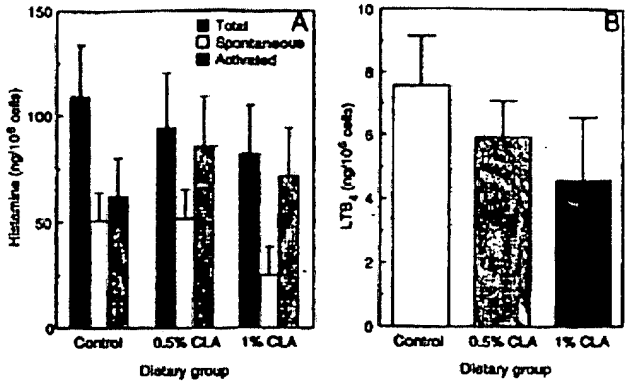

FIG. 1. Effect of dietary conjugated linoleic acid (CLA) on histamine content and release (A) and leukotriene B_4 (LTB_4) release (B) in rat peritoneal exudate cells. Means ± SE of five rats. Histamine release was measured in the presence and absence of calcium ionophore A23187. Total, total amounts of histamine in the cells; Spontaneous, the amount of histamine released during incubation without calcium ionophore A23187; Activated, the amount of histamine which was released from the cells when treated with A23187.

LTB_4 release in response to the dietary level of CLA, but the difference was not significant.

Tissue eicosanoid levels. The effect of CLA on LTB_4 and LTC_4 levels of spleen and lung is shown in Figures 2 and 3, respectively. CLA dose-dependently reduced the level of splenic LTB_4, and the difference between the control and 1% CLA groups was significant. No effect of CLA on the splenic LTC_4 level was observed. However, the concentration of LTC_4 in lung was reduced significantly by CLA even at the 0.5% dietary level. A trend of the dose-dependent reduction of LTB_4 also was observed, but the difference was not significant. The results of the levels of spleen and serum PGE_2 are summarized in Figure 4. CLA significantly reduced the concentration of serum PGE_2, while there was no effect of CLA on the splenic level of PGE_2.

Fatty acid compositions of PEC and splenic lymphocyte lipids. The PUFA composition of PEC and spleen lympho-

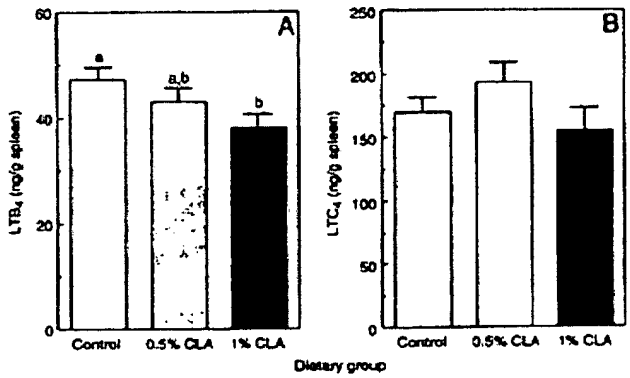

FIG. 2. Effect of dietary CLA on the concentration of splenic (A) LTB_4 and (B) leukotriene C_4 (LTC_4). Mean ± SE of five rats. Values without a common letter are significantly different at $P < 0.05$. For abbreviations see Figure 1.

TABLE 2
Effects of CLA on Growth and Tissue Weights of Rats[a]

Parameter	Group		
	Control	0.5% CLA	1.0% CLA
Initial body weight (g)	102 ± 1	101 ± 1	102 ± 1
Final body weight (g)	170 ± 2	166 ± 3	162 ± 4
Food intake (g/day)	19.1 ± 0.2	18.9 ± 0.3	18.6 ± 0.3
Tissue weight (g/100 g body weight)			
Liver	4.17 ± 0.09[a]	4.11 ± 0.09[a,b]	4.54 ± 0.07[b]
Kidney	0.85 ± 0.03	0.86 ± 0.03	0.87 ± 0.05
Perirenal adipose tissue	1.41 ± 0.07[a]	1.09 ± 0.09[a,b]	0.97 ± 0.14[b]
Heart	0.40 ± 0.02	0.34 ± 0.04	0.34 ± 0.04
Lung	0.48 ± 0.02	0.52 ± 0.02	0.49 ± 0.01
Spleen	0.22 ± 0.01	0.22 ± 0.01	0.25 ± 0.02
Brain	0.66 ± 0.02	0.70 ± 0.01	0.70 ± 0.01
Testis	0.96 ± 0.04	0.87 ± 0.10	1.00 ± 0.03

[a]Mean ± SE of 5 rats. Control group received 1.0% linoleic acid; 0.5% CLA group, 0.5% each of linoleic and CLA; and 1.0% CLA group, 1.0% CLA, respectively. Values without a common superscript letter (a,b) are significantly different at $P < 0.05$. For abbreviation see Table 1.

cyte total lipids is shown in Table 3. There was a dose-dependent reduction by dietary CLA of all n-6 PUFA, 18:2, 20:3, 20:4 and 22:4 in PEC lipids, while there was no difference in the proportion of n-3 PUFA, 22:6 among the groups. A clearer change in these n-6 PUFA was shown in splenic lymphocyte total lipids, and the reduction of 20:4n-6 was significant on a 1.0% CLA diet. Docosahexaenoic acid also tended to decrease with dietary CLA. The decreasing trend of all PUFA in CLA-fed rats was mainly attributable to a moderate increase in major saturated fatty acids, and oleic acid tended to decrease similar to PUFA (data not shown).

Serum thiobarbituric acid value. The concentration of thiobarbituric acid-reactive substance in serum was not modified by dietary CLA, and the values were within 4.1 to 5.5 ng/mL serum in all groups of rats.

Serum Ig levels. As shown in Figure 5, CLA increased the concentration of IgA, IgG and IgM, while decreasing that of IgE in serum. The difference between the control and 1.0% CLA groups was significant in these Ig.

Ig levels in spleen and MLN lymphocytes. Table 4 shows the Ig levels in the medium of rat spleen and MLN lymphocytes cultured for 72 h with or without lipopolysaccharide. Irrespective of the presence or absence of lipopolysaccharide, CLA showed no detectable effects on the Ig levels in spleen lymphocytes except for those of IgM after incubation with lipopolysaccharide, where CLA increased it in a dose-dependent manner. Under the similar situation, CLA increased the concentration of IgA, IgG, and IgM in MLN lymphocytes. The magnitude of the increase was particularly marked at the dietary CLA level of 1.0%. In contrast, there was a significant reduction of the IgE level when the cells from rats fed a 1% CLA diet were incubated with lipopolysaccharide in comparison with the control. A similar response to CLA also was observed even when these cells were incubated for 24 h (data not shown).

Subsets of MLN lymphocytes. The proportion of T-lymphocyte populations of MLN was analyzed as $CD4^+$ and $CD8^+$ subsets. There were no effects of CLA on their relative proportions ($CD4^+/CD8^+$ ratio; 2.6 ± 0.3, 2.4 ± 0.2, and 2.8 ± 0.1 for the control, 0.5% CLA, and 1.0% CLA, respectively).

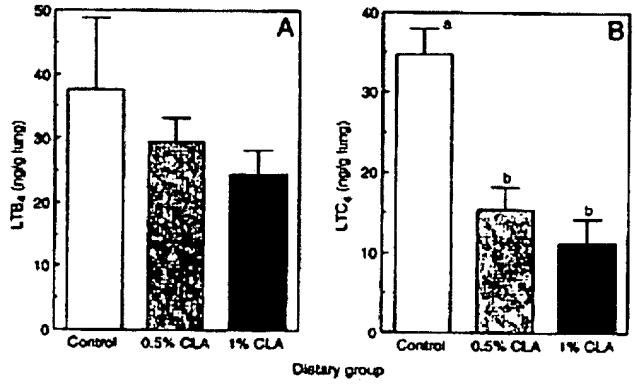

FIG. 3. Effect of dietary CLA on the concentration of lung (A) LTB_4 and (B) LTC_4. Mean ± SE of five rats. Values without a common letter are significantly different at $P < 0.05$. For abbreviations see Figures 1 and 2.

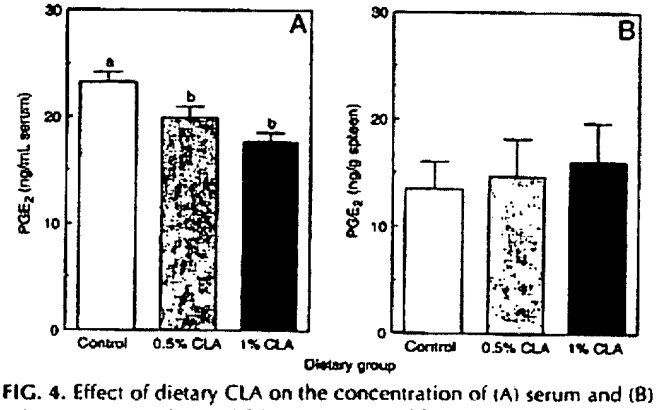

FIG. 4. Effect of dietary CLA on the concentration of (A) serum and (B) spleen prostaglandin E_2 (PGE_2). Mean ± SE of five rats. Values without a common letter are significantly different at $P < 0.05$. For other abbreviation see Figure 1.

TABLE 3
Effects of CLA on Polyunsaturated Fatty Acid Compositions of Peritoneal Exudate Cells and Spleen Lymphocyte Total Lipids of Rats[a]

Cells and fatty acid	Group		
	Control	0.5% CLA	1.0% CLA
	(wt%)		
Peritoneal exudate cells			
18:2n-6	5.5	5.3	4.2
20:3n-6	0.8	0.7	n.d.
20:4n-6	12.7	11.3	9.0
22:4n-6	5.6	5.3	4.2
22:6n-3	0.6	0.6	0.5
CLA			
9*t*,11*c*/9*c*,11*t*	n.d.	0.1	0.2
10*t*,12*c*	n.d.	0.2	0.2
Spleen lymphocytes			
18:2n-6	12.2 ± 0.8	10.4 ± 0.9	9.3 ± 0.9
20:3n-6	1.6 ± 0.2	1.3 ± 0.3	0.9 ± 0.1
20:4n-6	20.2 ± 0.8[a]	15.4 ± 1.3[a,b]	14.7 ± 1.7[b]
22:4n-6	2.5 ± 0.1	2.0 ± 0.2	1.9 ± 0.2
22:6n-3	1.2 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
CLA			
9*t*,11*c*/9*c*,11*t*	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
10*t*,12*c*	n.d.	0.2 ± 0.0	0.2 ± 0.0

[a]Values are means of two pooled samples from two and three rats each for the exudate cells, and means ± SE of three, five, and five rats for control, 0.5% CLA, and 1.0% CLA, respectively. Values without a common superscript letter (a,b) are significantly different at $P < 0.05$; n.d., not detected. For other abbreviation see Table 1.

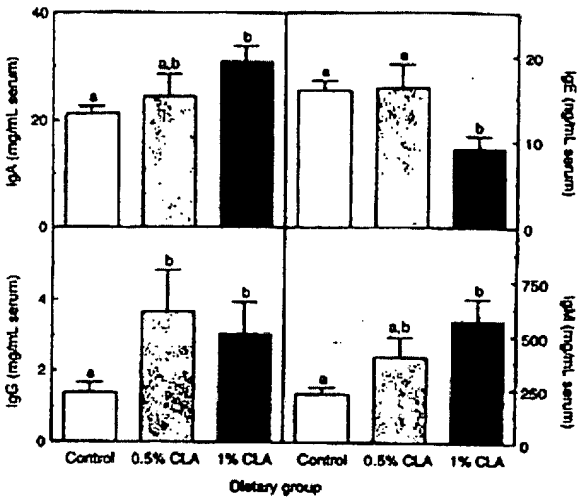

FIG. 5. Effect of dietary CLA on the concentration of serum immunoglobulins (Ig). Mean ± SE of five rats. Values without a common letter (a,b) are significantly different at $P < 0.05$. For abbreviation see Figure 1.

DISCUSSION

The pathway from linoleate to arachidonate and then eicosanoids is crucial to a range of metabolic diseases (28,29). Food allergy is one such disorder, and it is known that some eicosanoids are involved as chemical mediators in the manifestation of clinical symptoms of hypersensitivity (12,13). The inhibitors of LT production have now been clinically adopted (30,31). However, less is known of the effect that food components exert on this process. Although several food components have been shown to reduce eicosanoid production *in vitro*, in most cases it is practically unsatisfactory because of the limited efficacy (21,22). The results of the present study showed that CLA effectively controlled the production of LTB_4, LTC_4, and PGE_2. CLA significantly reduced LTC_4 production in the lung but not in the spleen. A similar tissue-specific reduction of LTC_4 was observed in rats given sesamin and α-tocopherol simultaneously, while in the spleen LTB_4 but not LTC_4 was reduced (21,22). These observations suggest a complex interaction between dietary fat and antioxidants in the LT-producing system.

Numbers of animal studies showed that dietary PUFA effectively modify the production of eicosanoids, and there is an interaction between n-6 and n-3 PUFA (32). PUFA of the n-3 family suppress the production of eicosanoids from arachidonic acid and exert a substantial suppressing effect on carcinogenesis in breast and colon (33,34). However, the anticarcinogenic effect of n-3 PUFA is far less than that of CLA (2–4). Eicosanoid production is known to be dependent on

TABLE 4
Effects of CLA on the Immunoglobulin Production in Splenic and Mesenteric Lymph Node Lymphocytes of Rats[a]

Immunoglobulin	Without lipopolysaccharide			With lipopolysaccharide		
	Control	0.5% CLA	1% CLA	Control	0.5% CLA	1% CLA
Spleen (ng/mL)						
IgA	3.75 ± 1.23	4.83 ± 0.99	3.78 ± 0.96	9.74 ± 2.45	13.6 ± 3.27	8.30 ± 2.50
IgG	51.0 ± 4.6	53.8 ± 2.3	61.5 ± 2.8	68.1 ± 2.4	71.9 ± 1.9	74.4 ± 1.9
IgM	223 ± 22	228 ± 6	246 ± 9	311 ± 9[A]	348 ± 8[B]	394 ± 6[C]
IgE	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Mesenteric lymph node (ng/mL)						
IgA	1.65 ± 0.13[a]	4.78 ± 1.77[b]	5.05 ± 0.10[b]	2.91 ± 0.23[A]	8.72 ± 0.90[B]	22.3 ± 0.7[C]
IgG	n.d.	3.08 ± 0.69[a]	28.1 ± 4.38[b]	n.d.	4.64 ± 0.11[A]	31.9 ± 4.1[B]
IgM	1.86 ± 0.34[a]	4.74 ± 0.50[a]	96.6 ± 13.4[b]	2.85 ± 0.44[A]	6.36 ± 0.48[B]	122 ± 9[C]
IgE	3.81 ± 0.32	4.02 ± 0.33	3.64 ± 0.47	4.81 ± 0.17[A]	4.52 ± 0.29[A]	3.74 ± 0.21[B]

[a]Means ± SE of five rats. Values without a common superscript letter (A,B,C,a,b,c) are significantly different at $P < 0.05$. The lymphocytes were incubated with or without lipopolysaccharide for 72 h, and the concentration of immunoglobulins (Ig) in the supernatant was measured; n.d., not detected.

the substrate availability (35). CLA reduced the proportion of n-6 PUFA including arachidonic acid in the immune cells as observed in the liver and other tissues (8,14). Because of the limited availability of PEC samples for fatty acid analysis, they were analyzed as two pooled samples from two and three rats each. Though the number of analysis may not permit us to draw a definite conclusion, it seems likely that fatty acid composition of PEC also responded similarly as in spleen lymphocytes. This reduction was at least responsible for the reduced production of LT and PG in these cells. CLA may affect metabolic interconversion of fatty acids in the liver that may ultimately result in modified fatty acid composition and arachidonate-derived eicosanoid production in extrahepatic tissue (14). However, more direct participation of the metabolites of CLA cannot be ruled out (9,36). Therefore, the present study added possible usefulness of CLA for controlling the allergic reaction caused by food. Since the effect of CLA on Ig production differed between MLN lymphocytes and spleen lymphocytes, the analysis of the fatty acid composition of the former cells may provide a clue to understanding the mechanism of action.

In contrast to the eicosanoid production, the level of histamine released from PEC, which reflects the mast cell degranulation by a receptor-independent pathway, apparently was not modified by CLA and more directly the fatty acid composition of membrane phospholipids. Engels *et al.* (37) observed that the type of dietary fats and thus the change in the fatty acid composition of mast cell phospholipids did not influence the cell degranulation process. CLA is reported to be incorporated into triglyceride more preferably than phospholipids in tumor cells (7). Thus, CLA may not substantially influence the fatty acid composition of membrane phospholipids and hence, the structure and function of the membrane. In such a situation, the degranulation of the mast cells may not be modified largely.

An interesting observation is that CLA regulates the Ig production class specifically. Food allergy reaction is initiated by the production of allergen-specific IgE (12,13). IgA, in contrast, serves as an antiallergic factor by interfering with the intestinal absorption of allergen, and IgG also works as an antiallergic factor through the competition with binding of allergen to the receptor on the surface of the target cells such as mast cells and basophiles (12,13). CLA increased the production of IgA and IgG, while reducing that of IgE in lymphocytes, in particular MLN lymphocytes irrespective of the presence or absence of lipopolysaccharide, a cell activator. The response of splenic lymphocytes to CLA was less clear except for a slight but significant increase in IgM after lipopolysaccharide activation. However, the response pattern similar to MLN lymphocytes was observed in serum, indicating that CLA can modify the Ig levels preferably even on a whole-body basis. Bile acids (24) and unsaturated fatty acids (25) also regulate antibody production class specifically, but in a manner contrasted from that of CLA. These compounds may promote the allergic response through an increase in IgE production and a reduction in IgA and IgG production. It is plausible that the production of IgE and of IgA and IgG are at least reciprocally regulated. Thus, in addition to the favorable effect on the eicosanoid production, CLA was expected to mitigate the food allergic reaction.

The amounts of CLA ingested by rats of the present study corresponded to approximately 30 and 60 mg/100 g body weight for 0.5 and 1.0% CLA diets, respectively. These amounts were pharmacological when extrapolated to human, 18 and 36 g/60 kg body weight/day. However, as in the case of weight reduction in man, approximately 3 g/d for 2 to 3 mon, a prolonged ingestion may produce a favorable effect even at a lower dose. A long-term trial with a lower dietary level of CLA merits further study.

In conclusion, CLA produced a situation favorable for mitigation of food allergic reaction. Since the effect was seen at a dietary level as low as 0.5 or 1.0%, it is likely that CLA can strongly regulate multiple metabolic processes. Thus, the clinical application of CLA is warranted. Studies with immunized animals will provide more direct information regarding this issue.

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